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Mechanism of Inhibition of Vaccinia Virus DNA Polymerase by Cidofovir Diphosphate

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Cidofovir (CDV) is a broad-spectrum antiviral agent that has been approved for clinical use in the treatment of cytomegalovirus retinitis. It has also been used off label to treat a variety of other viral infections, including those caused by orf and molluscum contagiosum poxviruses. Because it is a dCMP analog, CDV is thought to act by inhibiting viral DNA polymerases. However, the details of the inhibitory mechanism are not well established and nothing is known about the mechanism by which the drug inhibits poxvirus DNA polymerases. To address this concern, we have studied the effect of the active intracellular metabolite of CDV, CDV diphosphate (CDVpp), on reactions catalyzed by vaccinia virus DNA polymerase. Using different primer-template pairs and purified vaccinia virus polymerase, we observed that CDV is incorporated into the growing DNA strand opposite template G's but the enzyme exhibits a lower catalytic efficiency compared with dCTP. CDV-terminated primers are also good substrates for the next deoxynucleoside monophosphate addition step, but these CDV + 1 reaction products are poor substrates for further rounds of synthesis. We also noted that although CDV can be excised from the primer 3′ terminus by the 3′-to-5′ proofreading exonuclease activity of vaccinia virus polymerase, DNAs bearing CDV as the penultimate 3′ residue are completely resistant to exonuclease attack. These results show that vaccinia virus DNA polymerase can use CDVpp as a dCTP analog, albeit one that slows the rate of primer extension. By inhibiting the activity of the proofreading exonuclease, the misincorporation of CDV could also promote error-prone DNA synthesis during poxvirus replication.

Poxviruses are large, double-stranded DNA viruses that replicate in the cytoplasm of infected cells. Members of this virus family can cause severe infections, including human smallpox. Smallpox was declared eradicated in 1980, but concerns over bioterrorism (12, 27) and a recent outbreak of monkeypox in the midwestern United States (19) illustrate some need for the continued development of effective new treatment regimens. A number of new treatments are currently the subject of active investigation, including immunotherapy. However, antiviral drugs offer a combination of chemical stability and simplicity of delivery that is especially attractive from a public health perspective.

One class of drugs that have been shown to inhibit poxvirus replication are the nucleoside phosphonate analogs of cellular deoxyribonucleotides that were developed by De Clercq et al. (9). These drugs have been shown to be effective against a wide range of DNA viruses and retroviruses (reviewed in reference 8), and one of these compounds, (*S*)-1-[3-hydroxy-2-(phosphonylmethoxypropyl)]cytosine, also known as cidofovir (CDV), has been granted Food and Drug Administration approval for the treatment of cytomegalovirus (CMV)-induced retinitis. CDV has been used off label in the treatment of orf (11) and molluscum contagiosum (21) virus infections. It has also been shown to block the replication of variola and monkeypox viruses in culture (1) and to protect mice from a lethal challenge dose of ectromelia, vaccinia, or cowpox virus (2, 3).

Unfortunately, CDV causes significant problems of nephrotoxicity and can only be administered by intraperitoneal or intravenous injection. These problems have been addressed by the Hostetler group, who have shown that alkoxyglycerol or alkoxypropanediol esters of CDV and cyclic CDV are 50 to 230 times more active than CDV against vaccinia virus in vitro (17) while also being orally bioavailable (5). Further studies showed that this family of CDV analogs also blocked the replication of variola, monkeypox, cowpox, and ectromelia viruses in vitro (3, 15, 17) and when delivered orally protect mice from a lethal challenge with vaccinia, cowpox, or ectromelia virus (3, 22).

CDV is taken up into cells by fluid phase endocytosis (7), whereas its alkoxy derivatives are more rapidly absorbed through direct association with the lipid bilayer (5). Regardless of the uptake route, the alkoxyester link (if present) is then hydrolyzed to yield CDV and the CDV is phosphorylated in a two-step process to yield the active intracellular metabolite CDV diphosphate (CDVpp). The phosphorylation of CDV is carried out by cellular enzymes and in the absence of viral infection (6). This property of CDV results in drug efficacy even against kinase-deficient or mutant viruses. Human DNA polymerases α, β, and γ also exhibit some natural resistance to CDVpp, thus partially explaining the antiviral specificity of the drug (4, 14).

Previous studies have examined the effects of CDVpp on the activity of the CMV DNA polymerase (29, 30). This is a member of the B family of DNA polymerases, and it possesses both 5′-to-3′ polymerase and 3′-to-5′ exonuclease activities encoded

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within a single polypeptide. These studies have shown that CDVpp does behave as a dCTP analog, which CMV DNA polymerase can incorporate into a growing polynucleotide chain opposite template G's (29, 30). Under these circumstances, a single molecule of incorporated CDV somewhat retarded further DNA synthesis while adding two consecutive molecules of CDV opposite a pair of template G's caused a severe reduction or termination of elongation. In addition, a primer bearing a molecule of CDV at the 3′ terminus was refractory to the CMV enzyme's 3′-to-5′ exonuclease activity (29). These observations led Xiong et al. to suggest that a combination of these two enzymatic effects was responsible for the efficacy of CDV in CMV-infected cells (29).

These observations also suggest a mechanism by which CDV could inhibit orthopoxvirus DNA replication since poxviruses similarly encode B family DNA polymerases. The best characterized of the poxvirus polymerases is the 116-kDa E9L gene product encoded by vaccinia virus (10, 20, 25). This enzyme can be produced in a recombinant form (20) and serves as an excellent surrogate model for its variola virus homolog since the two enzymes differ by only 18 to 20 amino acids over a polypeptide spanning 1,006 amino acids. Vaccinia virus DNA polymerase normally forms part of a larger protein complex (16, 18), and its processivity is modified by the A20R virus gene product (18), but it is nevertheless catalytically active in isolation and this permits the characterization of its substrate specificity. In this study, we have used steady-state experimental methods and this purified form of vaccinia virus DNA polymerase to show that the effects of CDV on vaccinia virus DNA polymerase in vitro differ in important ways from the effects of the drug on betaherpesvirus DNA polymerases. These effects provide formal biochemical support for the contention that poxvirus DNA polymerases are an enzymatic target of these drugs. These studies also provide a starting point for the characterization of viruses exhibiting acquired resistance to CDV (24).

MATERIALS AND METHODS

Chemicals. CDVpp was synthesized by Trilink Biotechnologies, San Diego, CA. Radioactive nucleotides were purchased from Amersham Biosciences and oligonucleotides from Sigma-Genosys.

Vaccinia virus DNA polymerase. Recombinant vaccinia virus DNA polymerase was purified from vaccinia virus-infected cells according to the protocol of McDonald and Traktman (20) and appeared homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) analysis and silver staining (data not shown). The enzyme was stored at −20°C in glycerol at a concentration of 230 ng/μl. For use in enzyme assays, the enzyme was freshly diluted in 25 mM potassium phosphate (pH 7.4)–5 mM β-mercaptoethanol–1 mM EDTA–10% (vol/vol) glycerol–0.1 mg/ml bovine serum albumin (New England Biolabs).

DNA polymerase and exonuclease assays. Different combinations of oligonucleotide primers and templates were used to create different enzymatic substrates (Fig. 1). The primer strands were first end labeled using T4 polynucleotide kinase (Fermentas) and [γ-^{32}P]ATP, and the unincorporated nucleotides were removed using MicroSpin G-25 columns (Amersham Biosciences). Each 10-μl reaction mixture contained 1 pmol of end-labeled primer (approximately 90,000 cpm) and 3 pmol of template strand in a solution containing 30 mM Tris · HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, 80 μg/ml bovine serum albumin, and 0 to 25 ng of purified vaccinia virus DNA polymerase. Different concentrations of CDVpp and deoxynucleoside triphosphates (dNTPs) were added as indicated. The primer and template strands were mixed, heated to 55°C, and allowed to cool slowly to room temperature prior to adding the remaining reagents. The reaction mixtures were incubated at 37°C and stopped by adding 5 μl of gel loading buffer (80% formamide, 10 mM EDTA [pH 8.0], 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue). The reaction products were separated using a 10% denaturing polyacrylamide sequencing gel run at 45 W for 45 min in half-strength Tris-borate-EDTA (23). The gels were fixed in a solution containing 10% methanol plus 10% acetic acid and dried under vacuum. The dried gels were exposed to phosphorimager screens (Amersham Biosciences) and the images analyzed using a Storm 840 or Typhoon 8600 phosphorimager (Amersham Biosciences) with ImageQuant software (v. 5.1).

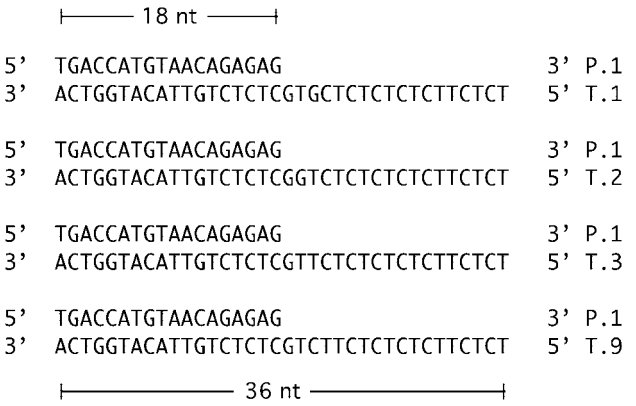

FIG. 1. Oligonucleotide primer-template pairs used in this study. The P1 primer and templates T1, T2, and T3 were originally described by Xiong et al. (29). Each primer strand was 5′ end labeled with ^{32}P prior to its annealing with a template strand. The melting point under the salt conditions used in this study (70 mM NaCl, 5 mM $MgCl_2$) is estimated to exceed 70°C. nt, nucleotides.

The large fragment of *Escherichia coli* DNA polymerase (Fermentas) and dideoxy sequencing reactions were used to generate size standards. The relative concentrations of each dideoxynucleoside triphosphate-dNTP pair were adjusted as needed to generate a suitable distribution of marker fragments.

Determination of K_m and V_{max} values for CDVpp. Standard 10-μl reaction mixtures were prepared as described above containing ~8 pmol of ^{32}P-labeled primer P1, 32 pmol of template T3, 25 ng of vaccinia virus DNA polymerase, 10 μM dGTP, and various concentrations of dCTP or CDVpp. After incubation for 0 to 16 min at 37°C, the reactions were stopped and the amount of primer molecules extended by 1 nucleotide was determined by PAGE and phosphorimager analysis. Initial primer extension rates were calculated for each CDVpp or dCTP concentration and K_m and V_{max} determined from a nonlinear regression fit of these data to the Michaelis-Menten equation. Prism software (v. 4.0b for Macintosh) was used to perform all the curve fits from which were obtained the relevant kinetic parameters. The results are presented as the mean ± the standard error of the mean.

RESULTS

Adding CDVpp to DNA polymerase reaction mixtures promotes chain termination. As a first step in determining the effect of CDVpp on vaccinia virus DNA polymerase, we performed a simple analysis of the rate of primer extension in the absence of any drug. These assay mixtures contained 10 μM each dATP, dTTP, and dGTP and 5 μM dCTP. The dNTP concentrations are similar to those estimated to exist in vivo in vaccinia virus-infected cells and vary from 2- to 10-fold greater than the calculated K_ms for each of the four nucleotides (13, 20). Figure 2 shows the results of one such assay. Vaccinia virus DNA polymerase has been estimated to incorporate dNTPs at a rate in excess of 30 s^{-1}, and under our steady-state conditions, the majority of the primers were extended out to near the end of the template strand in well under a minute (Fig. 2, top panel). We noted that the enzyme could never fully extend the primer strand out to the terminus of the duplex and created instead an array of products terminated 1 to 3 nucleotides from

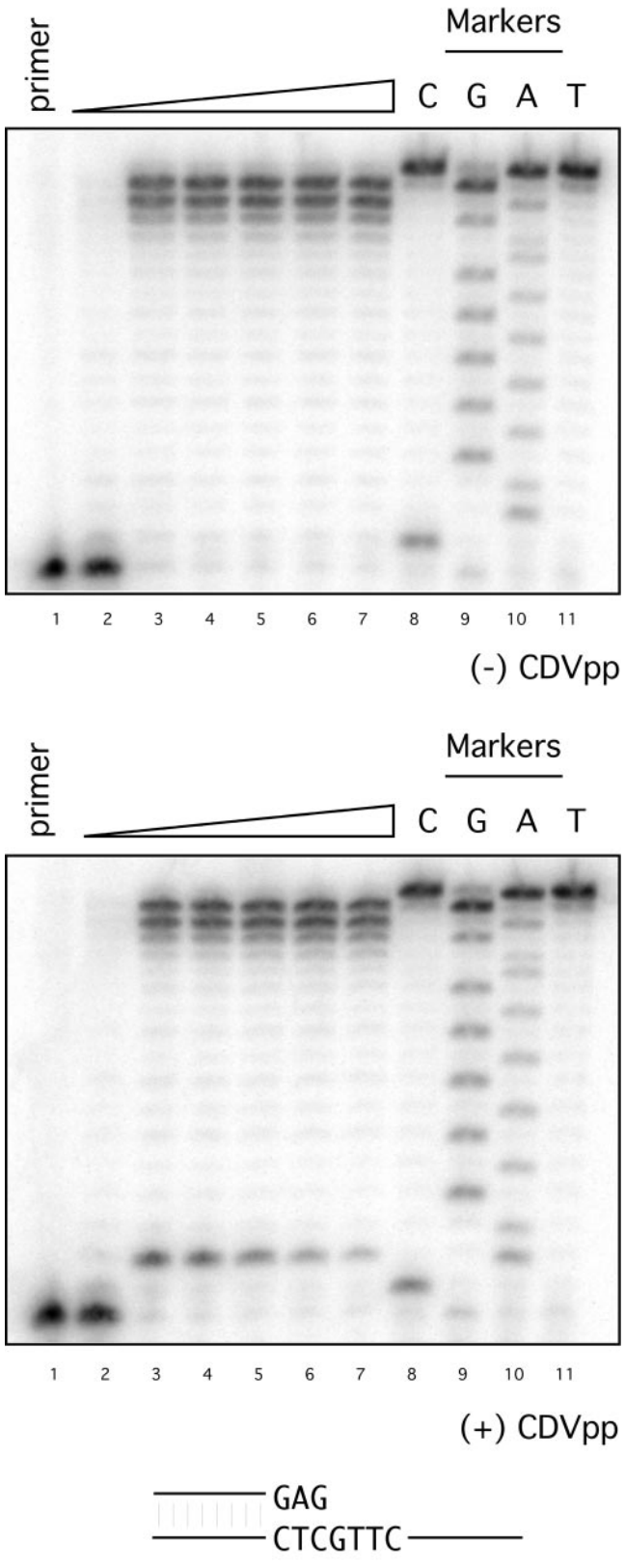

FIG. 2. CDVpp promotes chain termination in reaction mixtures containing a mixture of CDVpp and dCTP. Both reaction mixtures contained ^{32}P-labeled primer P1, template T3, and all four dNTPs (10 μM each dATP, dGTP, and dTTP plus 5 μM dCTP). The reaction mixture analyzed in the lower panel was further supplemented with 10 μM CDVpp. The reactions were started by adding vaccinia virus DNA polymerase, and the mixtures were incubated at 37°C. Sampling was conducted over a time scale ranging from 0 to 15 min, and each reaction was stopped by adding gel loading buffer on ice. The products were subjected to PAGE analysis, and ^{32}P-labeled molecules were detected by phosphorimaging. Note the accumulation of premature termination products in the lower panel. Comparison with dideoxy sequencing ladders (at right) shows that these reaction products are terminated 1 nucleotide past the point where CDV would be expected to be incorporated.

the end of the template strand, as judged by a comparison with the four sequencing lanes (Fig. 2). Small amounts of other partially extended strands were also sometimes detected at early times in the reaction. None of these effects were artifacts of the templates used in these studies and appear to be characteristic features of the nonprocessive form of vaccinia virus DNA polymerase. For example, a different polymerase (Klenow enzyme) extended essentially all of the primer strands out to the end of the template in reaction mixtures containing ddTTP (Fig. 2). (Note that the P1-T3 primer-template pair which was used in this reaction mixture contained no template dA's that could direct chain termination in the presence of ddTTP [Fig. 1].)

In contrast, adding 10 μM CDVpp to these reaction mixtures caused the accumulation of a significant fraction of products (28% at 1 min) terminated 2 nucleotides beyond the end of the primer strand (Fig. 2, bottom panel, lanes 3 to 7). When the size of these products is compared with the sequencing ladder, it is apparent that these CDV-induced stops are located at position N + 1, where N is the site where a molecule of CDV is expected to be incorporated opposite a template dG (Fig. 2). In contrast, strands that have avoided suffering this fate seem to have been extended out to the end of the template. It is important to note that this process does not irreversibly block DNA synthesis, because prematurely terminated molecules were slowly chased into full-length extension products over the 15-min time course of these experiments.

All of these studies were conducted using primer P1 annealed to template T3. To test whether these effects might be an idiosyncratic feature of this particular template-primer pair, we repeated the experiment using two additional oligonucleotide duplexes. Adding CDVpp to reaction mixtures containing a P1-T1 primer-template combination inhibited further chain extension after the primer had been extended by either 2 or 4 nucleotides (data not shown). Similarly, some termination was seen after primer P1 had been extended either 2 or 3 nucleotides when annealed to template T2 (data not shown). In all three situations, these termination sites were located at positions N + 1 relative to the site where a template dGMP would direct the incorporation of CDV.

CDV can be incorporated into DNA. These preliminary studies suggested that CDVpp promotes the termination of DNA synthesis but are complicated by the mixture of dCTP, CDVpp, and other dNTPs used in the reaction mixtures. We therefore performed a series of assays that examined each addition step in these primer extension reaction mixtures. To test the suitability of CDVpp as a substrate for vaccinia virus DNA polymerase, we investigated the kinetics of incorporation of CD-

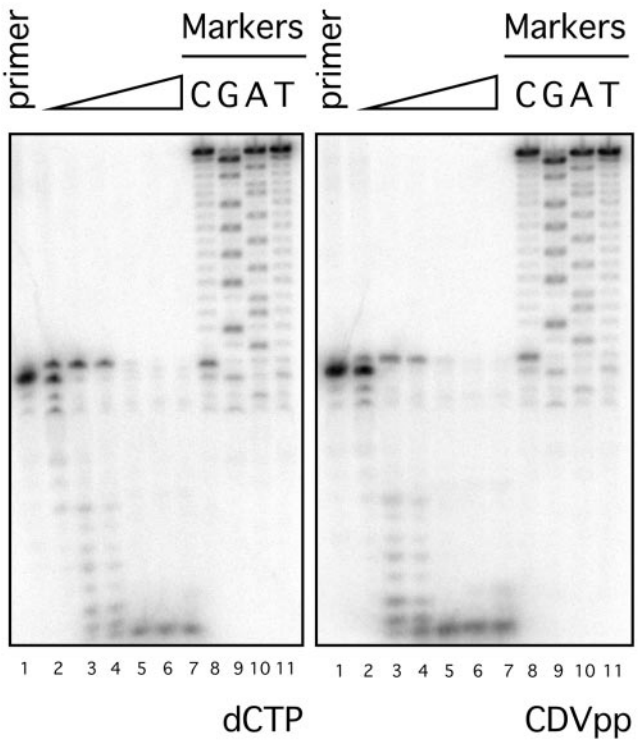

FIG. 3. Kinetics of dCTP versus CDVpp incorporation. Primer P1 plus template T9 was incubated with vaccinia virus DNA polymerase and either 10 μM dCTP (left panel) or 10 μM CDVpp (right panel). The reaction mixtures were sampled at 0, 1, 2, 5, 10, and 15 min; the reactions were stopped; and then the mixtures were subjected to PAGE analysis. Both compounds are rapidly incorporated into DNA during the initial stages of the reaction; at later time points, the primers are degraded into molecules as small as hexanucleotides.

Vpp versus dCTP using a combination of primer P1 and template T9. The reaction was supplied with only dCTP or CDVpp, so we expected the primer to be extended at most only 1 nucleotide beyond its 3′ end. The results of this experiment are shown in Fig. 3. Under these reaction conditions, we could not detect any great differences between CDVpp and dCTP as substrates for vaccinia virus DNA polymerase, with some conversion of the P1 primer into a primer + 1 product occurring in both reaction mixtures even on ice. By the first time point (1 min), the primer was quantitatively converted into the primer + 1 product in both reaction mixtures (Fig. 3, lanes 3). We also noted that by the 5-min time point molecules terminated with CDV or dCMP were broken down again into much smaller labeled reaction products (Fig. 3, lanes 5 to 7). Presumably, the 5′-to-3′ DNA polymerase and 3′-to-5′ proofreading exonuclease activities can consume all of the dNTPs in these reaction mixtures and, once this has happened, the proofreading exonuclease can then degrade the substrate down to a limit digest composed of hexanucleotides and other smaller oligonucleotides. CDVpp is a substrate for the polymerase activity, and CDV-terminated DNAs can be attacked by the proofreading exonuclease.

CDV-terminated primers are substrates for the vaccinia virus DNA polymerase. The pause sites detected in our primer extension assays containing CDVpp are located at positions N + 1 relative to the site of incorporation of CDV. This suggests that vaccinia virus DNA polymerase might encounter some difficulties utilizing CDV-terminated primers. To test this hypothesis, we first added dCMP or CDV residues to primer-template pair P1-T9 as described in Fig. 3. After sampling the reaction mixture, we added dATP and compared the subsequent rate of chain extension. The results are shown in Fig. 4. We noted that a dCMP-terminated primer was a good substrate for adding dAMP (Fig. 4, top, lanes 3 to 8), although a dynamic interplay between 3′-to-5′ proofreading exonuclease and polymerase activities was apparent and the enzyme eventually degraded the reaction products down to oligonucleotides over the course of the reaction. A CDV-terminated primer also directed the addition of dAMP with extension kinetics indistinguishable (using these methods) from a dCMP-terminated primer (Fig. 4, bottom panel). However, once extended by the addition of CDV plus dAMP, this reaction product was stable over the 15-min course of the experiment.

Primers bearing CDV as the 3′ penultimate base are poor substrates for vaccinia virus DNA polymerase. We next examined what happens when vaccinia virus DNA polymerase encounters a molecule bearing CDV as the penultimate 3′ residue. To do this, we used a P1-T9 primer-template pair and a series of sequential assembly reaction mixtures to incorporate CDV (10 μM CDVpp), followed by residue N + 1 (10 μM dATP), into DNA. We then tested the substrate properties of this primed structure after adding the remaining three dNTPs (no dCTP). As a control, dCTP was substituted for CDVpp. The results of these experiments are shown in Fig. 5. As noted in previous experiments (Fig. 4), vaccinia virus DNA polymerase produced a mixture of extension products terminated in dCMP or dCMP-dAMP (Fig. 5, top, lane 3). These two control reaction products were nearly instantaneously extended out to the ends of the template strand in the presence of dGTP, dATP, and dTTP (Fig. 5, top, lanes 4 to 9). Primers terminated in CDV-dAMP were synthesized in high yield (Fig. 5, bottom, lane 3), but the rate of extension of this product by vaccinia virus DNA polymerase clearly lagged well behind the rate of extension of control primers. Based upon the calculated distribution of label in substrates and products (data not shown), we estimated that the rate of primer extension, measured as a first-order rate constant, is at least threefold slower when CDV replaces dCMP as the 3′ penultimate nucleotide ($K = 3\ min^{-1}$ versus $0.9\ min^{-1}$ for extension from dCMP- versus CDV-bearing primers, respectively). However, there is a large standard error in the estimate of the control rate (95% confidence intervals for $K_{dCMP} = 0$ to $24\ min^{-1}$) and thus this calculation will probably underestimate the rate difference by at least a factor of 10.

We also looked to see if the enzyme could extend a primer incorporating two CDV molecules. Previous experiments with CMV DNA polymerase indicated that the presence of two consecutive CDV molecules dramatically reduced or terminated DNA synthesis (29). To compare the behavior of the two DNA polymerases, an assay was performed using primer P1 and template T2. The primer-template pair was first incubated with 10 μM CDVpp or dCTP (as a control), and then 10 μM dATP was added. Samples were taken for analysis after a brief incubation period, following by the addition of dATP, dGTP, and dTTP (10 μM each) to the remainder of the mixture. This

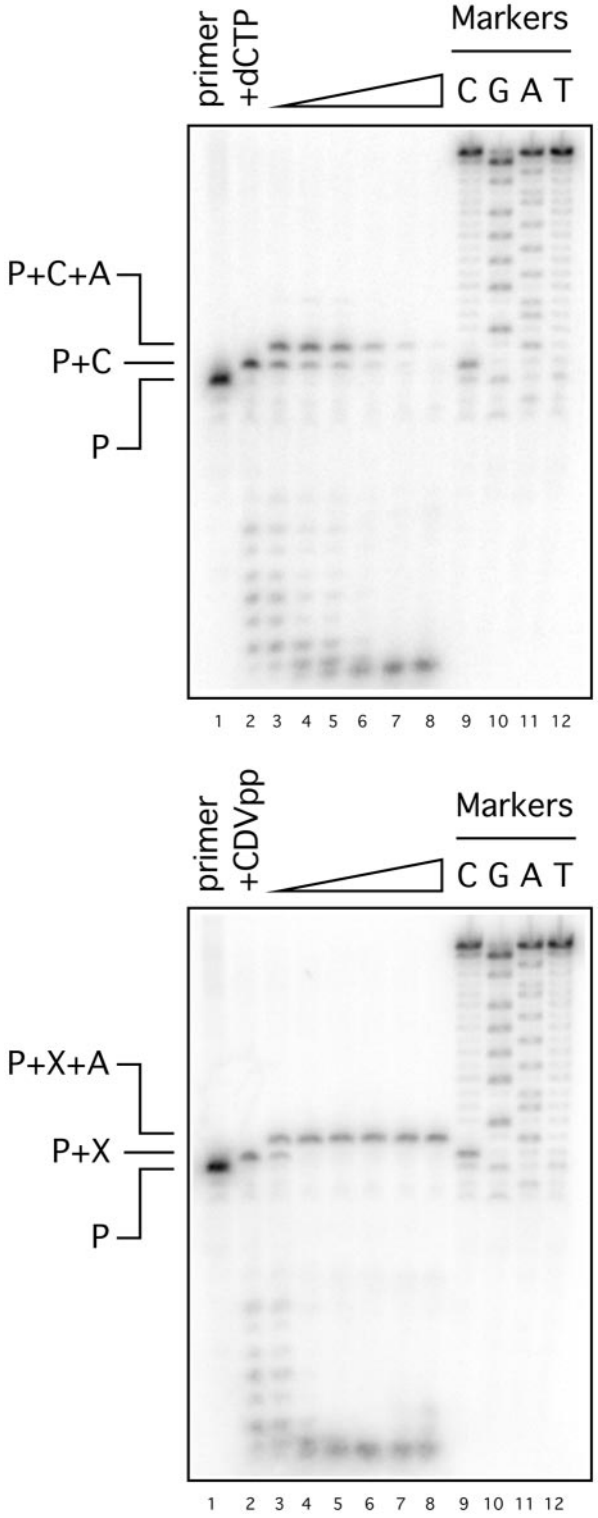

FIG. 4. Kinetics of dAMP addition to 3′-CDV- or 3′-dCMP-terminated primer strands. Duplex P1-T9 was incubated with vaccinia virus DNA polymerase and either 10 μM dCTP (top panel) or 10 μM CDVpp (bottom panel) for 1 min at 37°C. The concentration of dATP was then adjusted to 10 μM and further samples taken for gel analysis over another 15-min interval. The first samples (lanes 3) were taken on ice.

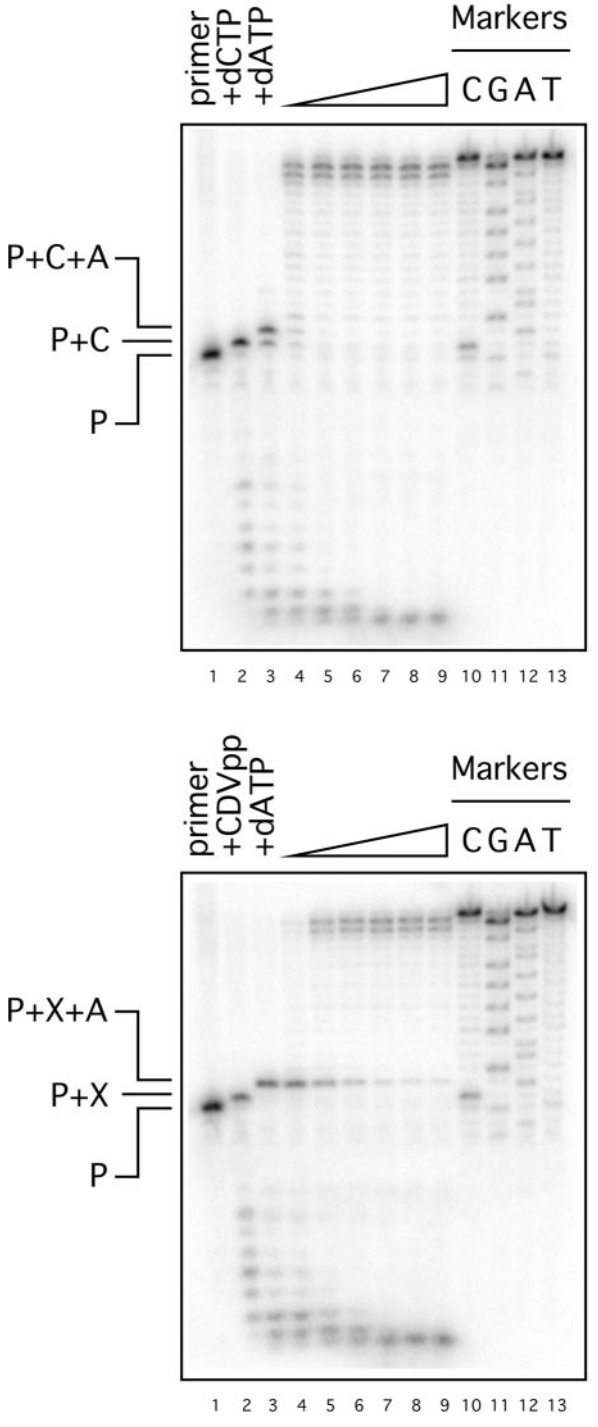

FIG. 5. Kinetics of chain extension using a 3′-CDV-dAMP-terminated primer. Substrates were prepared as described in the legend to Fig. 4, consisting of duplex P1-T9 that had been extended 2 nucleotides using dCTP-dATP (top) or CDVpp-dATP (bottom) as illustrated by Fig. 3 and 4. (Note that vaccinia virus DNA polymerase always generates a mixture of dCMP- and dCMP-dAMP-terminated products, whereas only a single CDV-dAMP product is formed.) A mixture of dATP, dGTP, and dTTP was then added and further samples taken for gel analysis over another 15-min interval. The first samples (lanes 4) were taken on ice.

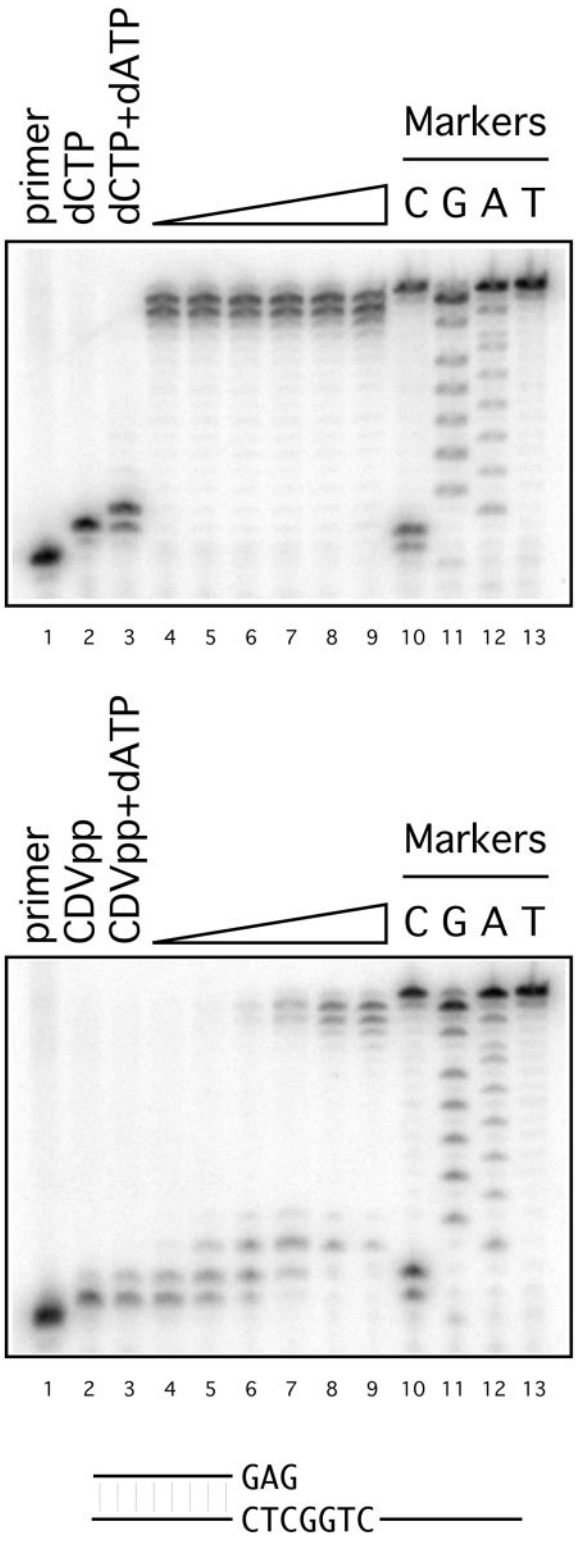

FIG. 6. The presence of two consecutive template dG's slows, but does not completely block, DNA synthesis. A series of reaction mixtures were used to incorporate dCMP-dAMP (top panel) or CDV-dAMP (bottom panel) onto the 3′ end of primer P1. This was accomplished by incubating P1-T2 with DNA polymerase plus 10 μM dCTP (top) or 10 μM CDVpp (bottom) for 1 min at 37°. After sampling the two reaction mixtures, dATP was added to a final concentration of 10 μM in each reaction mixture and the incubation continued for another 1 min at 37°C. Further samples were taken, and then a dNTP mixture was added to both reaction mixtures to produce final concentrations of 10 μM each dATP, dGTP, and dTTP with no additional dCTP. These solutions were incubated at 37°C with sampling at 1, 5, 10, 20, 40, and 80 min. A mixture of primers terminating in 5′-CC-OH 3′ or 5′-CCA-OH 3′ (top panel, lane 3) are fully extended to the end of the template strand in less than a minute by vaccinia virus DNA polymerase (lanes 4 to 9). However, the enzyme only reluctantly synthesizes molecules encoding two CDV resides (bottom panel, lanes 2 and 3) and takes much longer to assemble a primer terminating in 5′-XXA-OH 3′ (where X = CDV; lanes 4 to 9). These molecules do eventually chase into various amounts of full-length products over an 80-min period (lanes 7 to 9).

reaction mixture was then sampled over the next 80 min. The results are shown in Fig. 6. Molecules terminating with 5′-CC-OH 3′ and 5′-CCA-OH 3′ were again very rapidly extended out to the ends of the template within less than a minute (Fig. 6, top panel, lanes 4 to 9). In contrast, the rate of elongation of a primer incorporating two CDV molecules is drastically reduced, as judged by the very slow incorporation of the next (dAMP) residue and the subsequent extension of the primer out to the end of the template strand. However, it is also apparent that two CDV molecules do not completely block DNA synthesis catalyzed by vaccinia virus DNA polymerase. We saw the accumulation of primers terminated with 5′-XXA-OH 3′ and 5′-XXAG-OH 3′ (where X = CDV), but molecules extended immediately beyond this point were not seen and appeared to be chased into full-length products over the 80-min incubation time.

A single CDV molecule is a substrate for vaccinia virus DNA polymerase 3′-to-5′ exonuclease. Vaccinia virus DNA polymerase encodes a 3′-to-5′ proofreading exonuclease activity, and we also tested whether molecules terminated with CDV were substrates for this activity. To address this question, we first added a single dCMP or CDV residues to primer-template pair P1-T9 as described in Fig. 3. We purified these reaction products free of any unincorporated dCTP or CDVpp and then incubated the two extension products with fresh enzyme in the absence of any additional dNTPs or CDVpp. The results of this experiment are shown in Fig. 7. Molecules terminated with CDV (Fig. 7, bottom panel) were degraded as quickly as were dCMP-terminated primers (Fig. 7, top panel). This shows that a single CDV molecule is a substrate for the 3′-to-5′ proofreading exonuclease activity if it is located at the 3′ end of the primer strand.

The CDV + 1 product is not a substrate for the 3′-to-5′ exonuclease. In contrast to primers bearing a 3′-terminal CDV molecule, the CDV + 1 reaction product was completely resistant to attack by the proofreading exonuclease. For this assay, primer P1 and template T9 were again used. The primer-template pair was incubated together with 10 μM each CDVpp (or dCTP) and dATP, and vaccinia virus DNA polymerase, to create primers terminated with either 5′-CA-OH 3′ or 5′-XA-OH 3′ residues. The unincorporated dCTP, CDVpp, and/or dATP were removed by gel filtration, and then a new reaction mixture was prepared containing DNA polymerase but lacking any dNTPs (as above). Figure 8 shows the results of this experiment. Molecules terminated with a 5′-CA-OH 3′ sequence were rapidly attacked by the exonuclease, generating

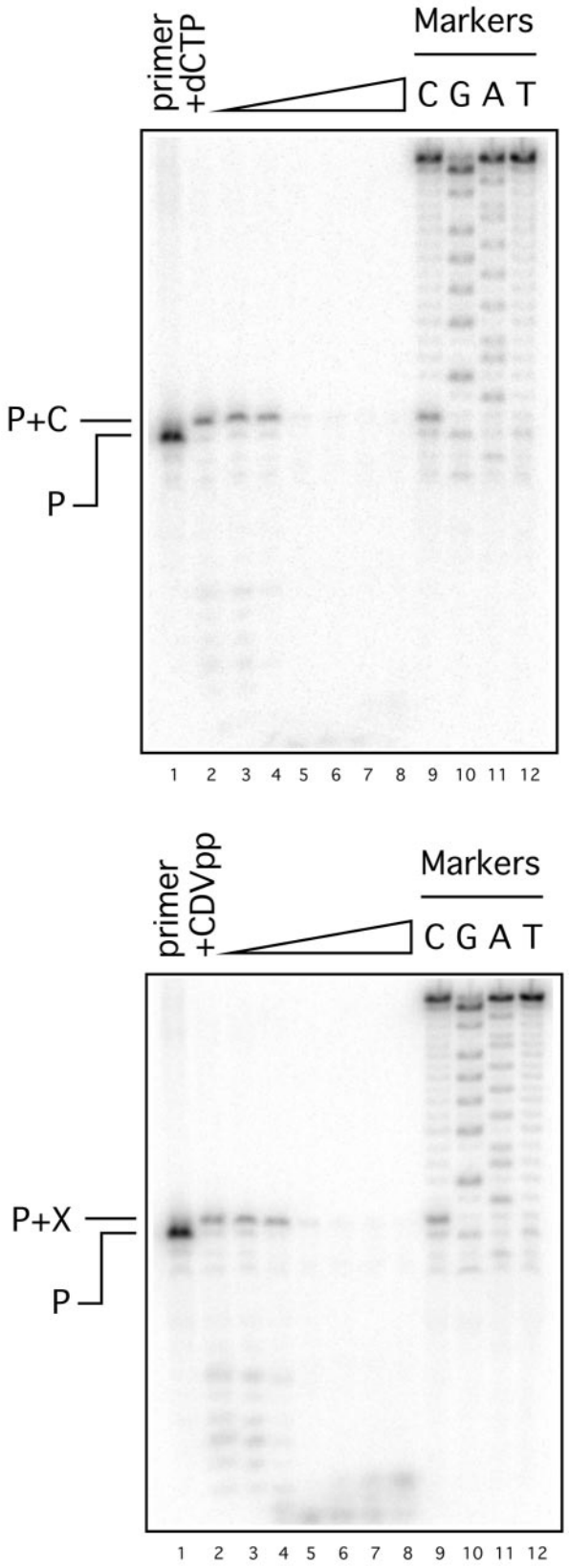

FIG. 7. Excision of CDV from a 3′-CDV-terminated strand. Duplex P1-T9 was extended 1 nucleotide using either dCTP (top) or CDVpp (bottom) and then purified free of reaction materials by gel filtration. The purified product was then incubated with vaccinia virus DNA polymerase at 37°C, in the absence of dNTPs, with sampling at 1, 5, 10, 20, 40, and 80 min. Both types of extension products are substrates for the 3′-to-5′ proofreading exonuclease.

a series of degradation products of which the smallest detectable by these methods were 6 to 7 nucleotides in length (Fig. 8, left panel, lanes 4 to 10). In contrast, the 5′-XA-OH 3′-terminated primer was stable over 80 min of incubation. A second molecule 13 ± 1 nucleotides long was also resistant to this activity (Fig. 8, right panel). These likely correspond to primer molecules that during the initial extension step (with CDVpp and dATP but without dGTP) were excised back to the next nearest 5′-CA-3′ residue. If this sequence motif had been replaced with 5′-XA-OH 3′, it would create a 13-mer molecule (Fig. 1) which, like the 20-mer, would also be resistant to nuclease attack. We concluded that the CDV + 1 extension product is resistant to attack by the vaccinia virus DNA polymerase 3′-to-5′ proofreading exonuclease.

Determination of the K_m of CDVpp. These observations provided a method for estimating the K_m of CDVpp. Reaction mixtures were prepared containing a P1-T3 primer-template pair, 25 ng of vaccinia virus DNA polymerase, and 10 μM dGTP. dGTP was added to maximize the stability of the dGMP-terminated primer in the presence of various concentrations of CDVpp. The kinetics of CDV incorporation were then determined in the presence of 0.3 to 30 μM CDVpp, monitoring the amount of labeled primer plus CDV extension product using polyacrylamide gel electrophoresis and phosphorimaging (Fig. 9). The various initial rates of CDV incorporation were then used to estimate that the K_m and V_{max} for CDVpp, under these reaction conditions, are 23 ± 6 μM and 3.0 ± 0.4 pmol · min^{-1}, respectively. Each reaction mixture contained 25 ng (0.22 pmol) of DNA polymerase and, assuming that the enzyme is fully active, the turnover value (k_{cat}) for CDVpp can be estimated as ~0.2 s^{-1}. The same methods were used to determine K_m (3.8 ± 0.7 μM), V_{max} (2.4 ± 0.2 pmol · min^{-1}), and k_{cat} (~0.2 s^{-1}) for dCTP (data not shown).

DISCUSSION

The effect of CDVpp on the activity of vaccinia virus DNA polymerase was investigated using an in vitro primer extension assay. Adding CDVpp to a pool of ordinary dNTPs caused the DNA polymerase to pause during chain extension at a point always 1 nucleotide past the site where CDV would be incorporated opposite dG. These observations provide a straightforward rationale for the antiviral activity against orthopoxviruses that has been reported in vitro and in vivo. They also explain why CDV-resistant viruses encode mutations in the E9L gene (DNA polymerase), mutations that can be shown to be responsible for the drug resistance phenotype by marker rescue methods (D.H.E. et al. and K.Y.H. et al., unpublished data).

Vaccinia virus DNA polymerase can use CDVpp as a substrate and can extend a CDV-terminated primer by 1 more nucleotide (Fig. 3, 4, and 9). The differences between the substrate properties of dCTP and CDVpp are not readily apparent from the steady-state data seen in Fig. 3, but a more detailed kinetic analysis clearly shows that CDVpp is a less

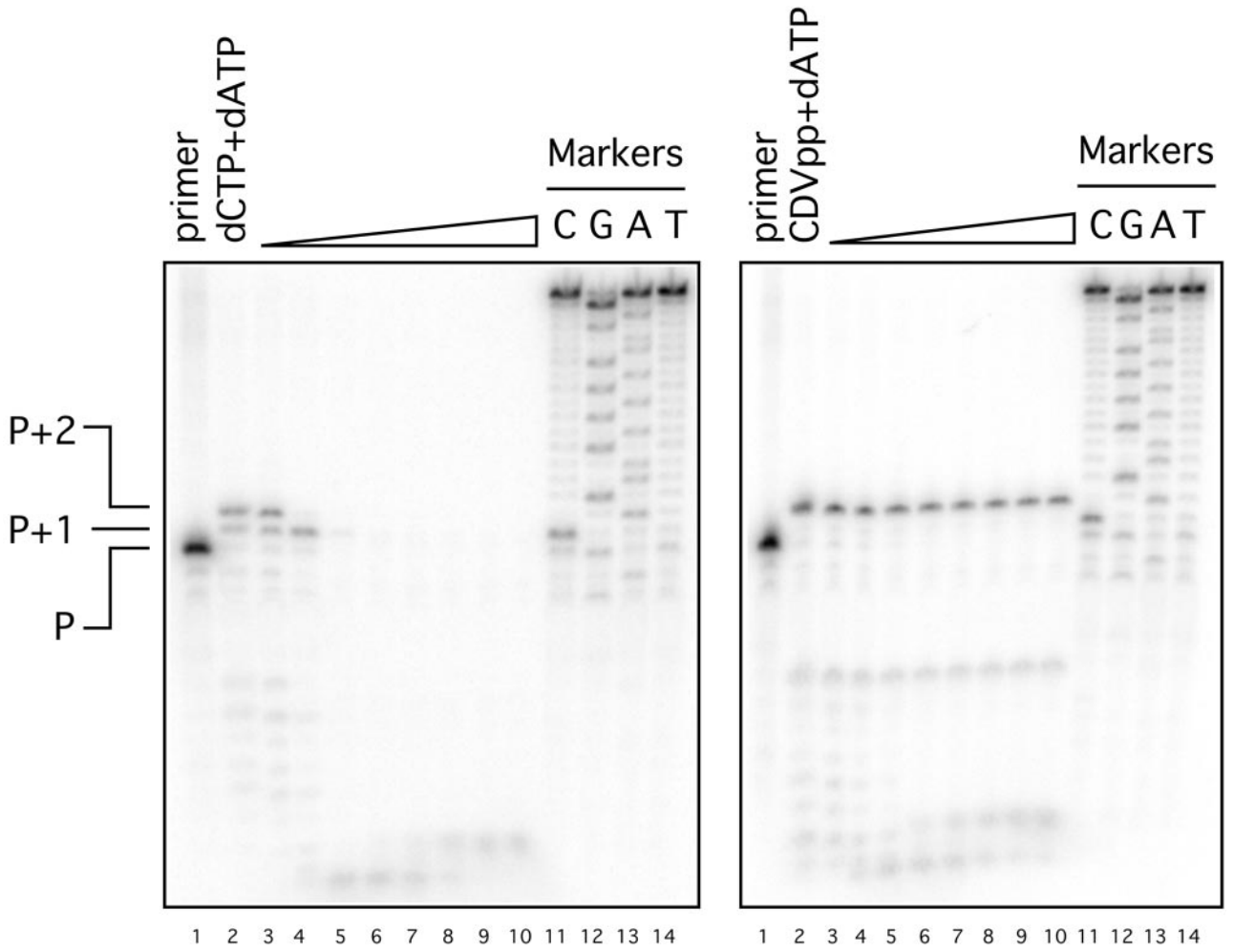

FIG. 8. CDV inhibits the 3′-to-5′ exonuclease activity of vaccinia virus DNA polymerase. Primer-template pair P1-T9 was incubated with vaccinia virus DNA polymerase at 37°C for 1 min in the presence of 10 μM dCTP and 10 μM dATP (left panel) or 10 μM CDVpp and 10 μM dATP (right panel). The unincorporated nucleotides and CDVpp were removed by gel filtration. The purified product was then incubated with vaccinia virus DNA polymerase at 37°C, in the absence of dNTPs, with sampling at 1, 5, 10, 20, 40, and 80 min. Molecules terminated with 5′-C-OH 3′ or 5′-CA-OH 3′ are rapidly degraded (left), whereas molecules terminated with 5′-XA-OH 3′ are completely resistant to exonucleolytic attack (right).

favored substrate than dCTP. This is illustrated by the fact that the catalytic efficiency is about fivefold higher for dCTP as a substrate versus CDVpp, with the difference primarily accounted for by differences in the K_m. The effects of CDV on the relative rate of addition of the next templated nucleotide (dATP) is more difficult to judge by these methods given the effects of the product on the proofreading exonuclease activity (see below) and the consequential biased accumulation of the CDV + 1 product. Nevertheless, the presence of a CDV molecule linked to the 3′ terminus of the primer strand still permitted the addition of the next nucleotide with kinetics superficially comparable to dATP addition to a dCMP-terminated primer (Fig. 4). CDV is thus not a chain-terminating drug in the classical sense, since it can be incorporated into DNA and still prime chain extension after incorporation into the nascent strand. From a structural perspective, CDVpp must be capable of occupying the nucleotide-binding site and serving as a substrate for the nucleophilic attack on the α-phosphonate. After translocation of the newly incorporated CDV molecule to the primer-terminus binding site, vaccinia virus polymerase must also be able to reposition the hydroxyl group with sufficient accuracy to permit further polymerization. The crystal structure of tenofovir complexed with human immunodeficiency virus reverse transcriptase illustrates the conformational flexibility of nucleoside phosphonates (26), which presumably accounts for this enzymatic behavior.

Although these substrate properties can partially explain the anti-poxvirus activity of CDVpp, more dramatic effects of the drug are seen at the next step in the polymerization cycle. Molecules bearing a CDV residue at the penultimate 3′ primer position are only very slowly extended into full-length chains (Fig. 5). The rate-limiting factor appears to be the reaction steps catalyzing the addition of the next nucleotide to the CDV + 1 product (Fig. 5). Although these molecules are poor substrates, they are still slowly converted into full-length products with a half-life of ~0.8 min under our conditions (data not

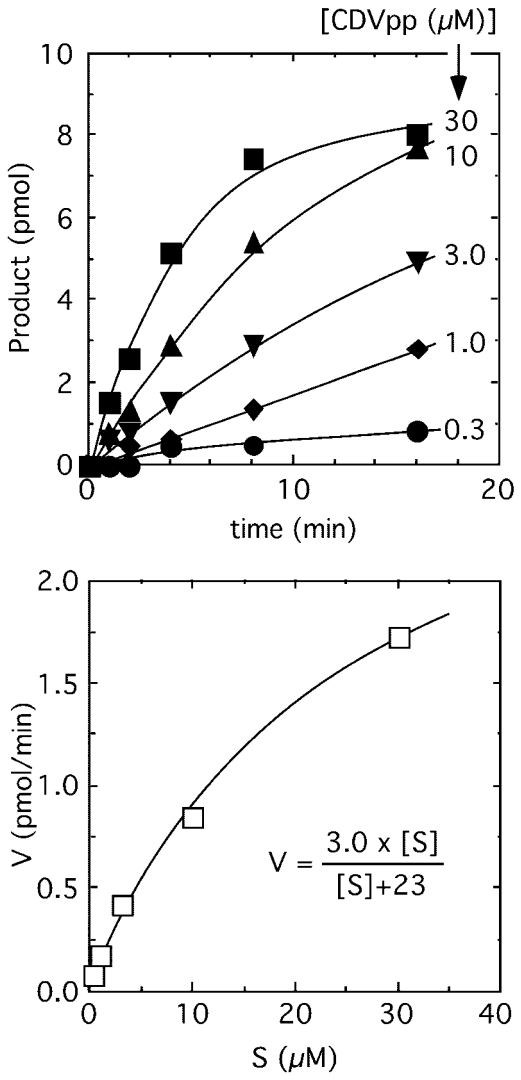

FIG. 9. Kinetics of CDVpp incorporation. The rate of CDV incorporation was determined using the primer-template pairs and electrophoretic methods shown in Fig. 3 and initial rates calculated from the data plotted as shown in the upper panel using nonlinear regression analysis and Prism 4.0 software (GraphPad). These data were replotted as shown in the bottom panel and the same nonlinear regression method used to calculate the Michaelis-Menten constants K_m (23 ± 6 μM) and V_{max} (3.0 ± 0.4 pmol · min^{-1}).

shown). Generally, the same effect was seen when two molecules of CDV were incorporated into the primer strand. However, the transient accumulation of a $CDV_2 + 2$ product and the much-delayed appearance of full-length product (Fig. 6) suggested that the deleterious effects of CDV are propagated further and with greater effect under such circumstances. Base-paired deoxynucleoside monophosphates are relatively inflexible molecules, and this property, combined with structural distortions imposed by a nucleoside phosphonate linkage, could result in misalignment of the 3′-hydroxyl terminus with deleterious effects on the next nucleotide addition step. Alternatively, the enzyme might have difficulty repositioning the CDV + 1 reaction product within the polymerase reaction site, which would also inhibit further chain extension.

The same effects are seen when one examines the susceptibility of CDV-containing molecules to attack by the 3′-to-5′ proofreading exonuclease. CDV is excised from the 3′ end of the primer at a rate comparable to dCMP (Fig. 7) and shows that vaccinia virus polymerase has little difficulty hydrolyzing a phosphonate ester linkage. This is not true of the CDV + 1 substrate, where the 3′-terminal (−1) phosphodiester linkage is completely resistant to hydrolysis (Fig. 8). These observations can be rationalized using the same structural arguments outlined above. CDV-terminated molecules might exhibit sufficient conformational flexibility to still serve as exonuclease substrates, while the constraints imposed by a phosphonate linkage at the −2 bond may render the phosphodiester bond at −1 resistant to hydrolysis.

HCMV DNA polymerase exhibits some of the same enzymatic responses to CDVpp (29, 30). In particular, the two different virus enzymes utilize CDVpp less efficiently that dCTP (30). Substituting CDVpp for dCTP also causes the transient accumulation of the CDV + 1 reaction product. However, poxvirus polymerases appear capable of slowly copying templates encoding a dGpdG motif in the presence of CDVpp (Fig. 6), while these residues completely block readthrough catalyzed by HCMV polymerase. A much more notable difference is that HCMV polymerase cannot excise CDV from a CDV-terminated primer (29), whereas it is the CDV + 1 reaction product that creates the greatest difficulties for vaccinia virus DNA polymerase. These differences suggest that the two enzymes interact with CDV and CDV-bearing DNAs in sometimes different ways, and caution must be observed extrapolating from the molecular genetic properties of CDV-resistant herpesviruses to poxvirus systems.

In conclusion, these data provide insights into the anti-poxvirus activity of nucleoside phosphonate drugs. These compounds inhibit chain extension, and since DNA synthesis is a key regulator of the virus life cycle, they are expected to compromise a diverse array of other processes, including intermediate and late gene transcription and virus assembly. The incorporation of CDV into DNA also completely inhibits the associated 3′-to-5′ exonuclease. The proofreading exonuclease serves a critical role in minimizing replication errors and probably also catalyzes virus genetic recombination (28, 31). Future studies will use CDV, and CDV-resistant viruses, as a tool for investigating these two critical viral systems.

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